Exploring the anti-aging and nootropic potential of aromatic essential oils using *Caenorhabditis elegans* models

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BY

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Private University Established in Karnataka by ACT 35 of 2013
BENGALURU – 560064

DECLARATION BY THE CANDIDATE

I declare that this thesis "**Exploring antiaging and nootropic potential of aromatic essential oils using *Caenorhabditis elegans* models**." submitted for the award of Master of Science to THE UNIVERSITY OF TRANS-DISCIPLINARY HEALTH SCIENCES AND TECHNOLOGY, Bengaluru, is my original work, conducted under the supervision of Dr. Ashwini Godbole. I confirm that no part of the work reported herein has been submitted for a degree or examination at any other university. References, funding and material obtained from other sources have been duly acknowledged, and no part of this dissertation has been plagiarised.

Place: TDU, Bengaluru

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CERTIFICATE FROM THESIS SUPERVISOR

This is to certify that the work incorporated in this thesis **"Exploring antiaging and nootropic potential of aromatic essential oils using *Caenorhabditis elegans* models."** submitted by Dr. Shachi Suvas Prabhu Mahambre was carried out under my/our supervision. No part of this thesis has been submitted for a degree or examination at any other university. References, help and material obtained from other sources have been duly acknowledged. I confirm the originality of the work and that there is no plagiarism in any part of the thesis.

Dr. Ashwini Godbole

Associate Professor

19/06/2024

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SUMMARY

In Ayurveda, selected *Sugandhi Dravya* (aromatic drugs) are traditionally used to treat depression disorders. This study focused on four essential oils *Chandana, Karpura, Vacha*, and *Jatamansi*, which were selected on their therapeutic benefits. Utilizing the *Caenorhabditis elegans* models, we assessed the nootropic and anti-ageing properties of these essential oils. Additionally, we investigated their effects on Ser-1 knockout mutant strain, which is defective in serotonin signalling and serves as a model for depression. Our research examined the overall lifespan, healthspan, fecundity and oxidative stress in these worms.

The findings revealed that *Chandana* essential oil demonstrated the most significant positive effects among the tested oils. It extended the mean lifespan and improved the health span of Ser-1 knockout *C. elegans*. These results suggest that *Chandana* possesses strong nootropic and anti-ageing properties, making it a promising candidate for further studies on depression and age-related disorders. The study underscores the potential of Ayurvedic aromatic drugs in modern therapeutic applications.

PERSONAL REFLECTION

When I started this journey as a novice researcher, little did I know that 'the worm' would become an important part and parcel of my academic journey. I learnt so many things while working on my thesis over the last 8 months. First and foremost, I understood the importance of slowing down and developing ample patience. Picking of worms requires both of the above.

Technically, I learnt how to work in a lab and handle instruments. I also learnt how to prepare reagents. I learnt to let go off the experiment batches that did not go as per the required standards of the experiments I ran. I learnt to be unbiased and to have a positive attitude. I understood the basics of preliminary data collection and the challenges that come with it.

While it is established in the classics of Ayurveda, that *Sugandhi dravyas* have an array of functions it was gratifying to see the same with the results of the experiments. I would like to pursue this further to get a more in-depth understanding of the same.

While doing Sensory analysis on human volunteers and analyzing data as also challenging.

Working on this thesis definitely widened my horizon and my social circle. I got to delve into the universe of this mighty tiny creature which is commonly referred to as ‘the worm’ in the world of research.

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1. INTRODUCTION

1.1 Sugandhi Dravya (Aromatic Drugs)

According to Ayurveda, the *panchendriya* (5 sense faculties) help to gain knowledge from the surroundings. Amongst five, *Ghranendriya* (Olfactory system) is responsible for sensing odour cues via the nose. This cue is passed from *manas* (mind)- to *buddhi* (intellect) to *atma* (soul) and the smell is perceived. *Ghranendrya* is also used as a modality to deliver medicines for wellness as well as various medical conditions. The medicines can be in the form of *Choorna* (powder*), dhuma* (fumes) and *taila* (oils) which have *Sugandha* (good smell) or *durgandha* (bad smell).

The definition of *Sugandha* and *durgandha* is,

"सुखानुबन्धी सूक्ष्मश्च सुगन्धो रोचनो मृदुः ॥८।

दुर्गन्धो विपरीतोऽस्मात् हृल्लासारुचिकारकः ।९।"

Sugandha is a pleasant fragrance; whereas *durgandha* has the opposite qualities. In the commentary*, Acharya Shivdas Sen* from the 14th century further explains the properties of pleasant fragrances as, "*Sukhanubandhi"* associated with happiness or joy, "*Sukha-janaka*" generates happiness and is subtle. The properties of unpleasant odours are undesirable, are associated with sorrow, give a contrary effect to pleasant fragrances and cause aversion.

Definition of health according to ayurveda is-

समदोषः समाग्निश्च समधातु मलक्रियाः।
प्रसन्नात्मेन्द्रियमनाः स्वस्थः इत्यभिधीयते ॥

(Prof. Vasanth C. Patil, 2018)

For a human to be called Healthy or *Swastha*, homeostasis has to be maintained between the *tridoshas, agni, dhatus* and they have to have a regular bowel movement and their soul mind and mind should be in a pleasant state.

Ayurveda has mentioned that the state of mind is also an indicator of health. According to previous studies by (Kontaris et al., 2020; Masuo et al., 2021) aroma has stress-reducing properties and has a direct effect on emotions. So, to investigate whether the *Sugandhi Dravya* mentioned in Ayurveda helps in wellness, a literature review was done and four drugs were selected. The four drugs were selected as they are commonly available and are cost-effective. Some of them are widely used in Indian households regularly. With recent advancements and awareness, aromatherapy is commonly used for healthcare and there is high acceptability.

The aromatic drugs used in this study are-

a. *Acorus calamus* Linn
   Sanskrit name- *Vacha*
   English name- sweet flag
   Family- *Araceae*
   IUCN status- Least Concern [LC] (IUCN 2017)
   Part used- Rhizome

b. *Cinnamomum camphora (L.) Nees & Eberm.*
   Sanskrit name- *Karpura*
   English name- Camphor
   Family- *Lauraceae*
   IUCN status- Least Concern [LC] (IUCN 2017)
   Part used- Resinous exudate

c. *Santalum album* Linn
   Sanskrit name- *Chandana*
   English name- Sandalwood
   Family- *Lauraceae*
   IUCN status- Vulnerable
   Part used- heartwood (*kanda sara*)

d. *Nardostachys Jatamansi (D.Don) DC*

Sanskrit name- *Jatamansi*

English name- Spikenard

Family- *Valerianaceae*

IUCN status- Critically endangered

Part used- Rhizome

According to Ayurveda, the classification is done based on the qualities of substances *rasa* (taste), *guna* (qualities), *virya* (potency), *vipaka* (transformation of taste after food) and *Prabhava* (distinct activity) are mentioned in table 1.1. *Sugandhi dravyas* have been mentioned in both *Brihatrayi* and *laghutrayi,* as part of treatment regimen for various disease conditions. Below are the uses of the selected drugs used in *Dinacharya* (daily regimen), *ritucharya* (seasonal regimen) and for treatment.

Essential oils

For over a decade, essential oils have been popular in the market (Manion & Widder, 2017). They are used in various products such as cosmetics, food, candles, soaps, lotion, oil, perfumes, etc (Sharmeen et al., 2021). They are complex substances with hundreds of bioactives which can vary largely during the extraction process. In this study, the EOs of the above-mentioned drugs were taken.

	Drug name	Rasa	Guna	Virya	Vipaka	Prabhava	Uses mentioned
I.	Vacha	Katu (pungent), Tikta (bitter)	Laghu(light), teekshna(strong)	Ushna (hot)	Katu (pungent)	Medhya (memory enhancing and intellect promoting)	Pachana (digestion), deepan (appetizer), Medhya (nootropic), jeevani (prolongs life), vak-prada (beneficial in speech), swaraprada (beneficial in voice), unmadahara (antipsychotic), apasmarahara (antiepileptic), anilhara (reduces vata)
II.	Karpura	Tikta(bitter), Katu (pungent), Madhura (sweet)	Laghu(light), Ruksha(dryness)	Sheeta (cold)	Katu (pungent)	-	Wellness: In grishma ritu (Summer) have it along with edible substances and use curtains sprinkled with fragrant drugs and water- as it cools the body; In Varsha ritu (rainy season) clothes are fumed with Karpura In Sharad ritu (Autumn) anointing the body with paste kanta doshahara (effective in throat diseases), vishahara (antitoxic), chakshushya (beneficial for eyes), madakaraka (causes intoxication), yogavahi (one which accelerates properties of others), dahahara (reduces burning sensation), vrushya(aphrodisiac), Medhya (nootropic), kriminasha (anthelmintic)
III.	Jatamansi	Tikta(bitter), Kashaya (astringent), Madhura (sweet)	Laghu(light), Snigdha (unctuousness)	Sheeta (cold)	Katu (pungent)	bhutaghna, manasa doshahara	medhya, balya (increasing strength), kushthaghna (corrects skin ailments), kandughna (relieves itching sensation), jwaraghna (antipyretic), dahahara, kanti karaka (improves complexion), tridoshahara (balances 3 doshas)
IV.	Chandana	Tikta (bitter), Madhura (sweet)	Laghu(light), rooksha (dryness)	Sheeta (cold)	Katu (pungent)	-	Wellness: In grishma ritu (Summer) and Sharad ritu (Autumn) anointing the body with a paste varnya, dahaprashamana, jvarahara, kushtahara, kandughna, trushnahara(quenches thirst), chardoghna(antiemetic), pradarahara (beneficial in menorrhagia)

Table1.1 *Rasapanchaka* of drugs along with their uses as mentioned in samhitas

Ayurveda categorises drugs based on their qualities used for the treatment in various medical conditions which are referred to as *ganas*. The four drugs used in this study are mentioned in different *ganas* as follows (Harinarayan Sharma, n.d.)

Gana	Use/proposed activity	***Vacha***	***Karpura***	***Chandana***	***Jatamansi***
Chardana/ Vamaka gana	For emetics	+			
Niruhana gana	For decoction of enema	+			
Pittaghna gana	Decreases pitta			+	
Sarivadi gana	Cures burning sensation, bleeding disease, thirst and fever			+	
Anjanadi gana	cure diseases due to poison, burning sensation inside the body, and balance pitta				+
Patoladi gana	balance Kapha-Pitta and cure skin diseases, fevers, poison, vomiting, anorexia and Jaundice			+	
Asanadi gana	cures leucoderma, skin diseases, mitigates kapha, cures diseases and other skin diseases, mitigates kapha, cures diseases caused by			+	

	worms, anemia, diabetes and diseases of fat accumulation				
Vatsakadi gana	cures disorders of vata, kapha and medas, rhinitis, abdominal tumour, fever, colic, and haemorrhoids	+			
Vachaharidradi gana	cures acute diarrhoea - caused by accumulations of ama diseases of fat, kapha, stiffness of the things and disorders caused by breast milk	+			
Priyangu ambashthadi gana	Good for pitta to decrease and heal ulcers			+	
Mustadi gana	Cures diseases related to vagina, breastmilk and does mala pachana	+			
Kandughna gana (Dharkar Nilima, 2023)	Cures spectrum of skin diseases			+	+

Table 1.2 Drugs mentioned in various ganas and their proposed activity. '+' indicates presence.

1.2 Depression Disorder

Depression is a prevalent mental health disorder that impairs psychosocial functioning and reduces quality of life, affecting approximately 280 million people worldwide in 2023, with around 5% of the global adult population suffering from it, according to the WHO (WHO, 2017). It has a huge impact on individuals and imposes an immense economic burden as it is a challenge to diagnose and manage depression (Malhi & Mann, 2018).

Symptoms

In order for a patient to be clinically diagnosed as depressed, they should have experienced irritability, sadness, emptiness, loss of pleasure or interest in activities for major parts of their day, every day for a period of two weeks. They may also present with other symptoms which include impaired attention, overwhelming feeling of guilt, low self-esteem, hopelessness about their future, disturbed sleep, changes in weight and feeling of low energy levels. (Malhi & Mann, 2018; Rakel, 1999)

Management & Treatment

The management of depression varies on the prognosis of the disease. Before treatment, the patient has to stop substance abuse and start with general hygiene, self-care, regular exercise, a healthy diet, etc. The management strategy includes Psychosocial, Psychological therapy, Pharmacotherapy and Electroconvulsive therapy. The line of treatment is based on the symptoms of the patient and how they respond to the therapy.

1. Psychosocial management includes Psychoeducation – family, friends, and caregivers, low-intensity interventions (eg, internet-based education), formal support groups, employment, and housing.

2. Psychological therapy includes cognitive behavioural therapy, interpersonal therapy, acceptance and commitment therapy and mindfulness-based cognitive therapy.

3. Pharmacotherapy includes First line- Selective serotonin reuptake inhibitors (SSRIs), Noradrenergic and specific serotonergic antidepressants (NaSSAs), Norepinephrine and dopamine reuptake inhibitors (NDRIs), or Serotonin and norepinephrine reuptake inhibitors (SNRIs); melatonin agonist, serotonin modulator and second line – Tricyclic antidepressants, MAOIs.

4. Electroconvulsive therapy- it is given to cases of severe depression when the first line of pharmacotherapy does not work (Malhi & Mann, 2018; Rakel, 1999)

SSRIs and SNRIs are associated with a range of side effects, including dizziness, fatigue, headaches, loss of appetite, weight loss, drowsiness, increased suicidal thoughts, nausea, vomiting, sexual dysfunction, and increased risk of cardiovascular and cerebrovascular incidents(Wang et al., 2018).

Relation between depression and serotonin

Serotonin, or 5-hydroxytryptamine (5-HT), is a monoamine neurotransmitter synthesized from the amino acid tryptophan (Liliana Dell'Osso, 2016). The Serotonin hypothesis proposes that diminished activity of serotonin pathways plays a role in the pathophysiology of depression. The major therapeutic agents for treating depression are antidepressants, mostly selective serotonin reuptake inhibitors (SSRI) or combined serotonin/noradrenaline reuptake inhibitors (Cowen & Browning, 2015)

Relation between depression and ROS

Chronic stress is one of the leading causes of depression. Chronic stress can lead to behavioral and genetic changes (Schmidt et al., 2008) Chronic stress is one of the leading causes of depression and mental disorders. Chronic stress can cause behavioral and genetic changes. Aside from psychological pressures, oxidative stress plays a significant effect. In certain investigations, oxidative stress marker levels differed between depressed patients and healthy people. Oxidative stress causes DNA and protein damage, as well as lipid peroxidation, all of which contribute to associated diseases including cardiovascular disease. Therefore, it may be suggested that antidepressant medications must fulfil various roles, i.e., lowering oxidative stress and

reactive oxygen species (ROS) formation and boosting levels of neurotransmitters, such as serotonin and dopamine (Naß et al., 2021).

3. *Kaphaja Unmad* and Depression

Unmada (a wide term that encompasses a variety of psychiatric issues) is a serious psychiatric disorder mentioned in the Ayurvedic classics as having distorted mental functioning. *Unmada*'s etymological meaning is "a state of disturbed mental functions." According to *Acharya Charka*, the characteristic pathological feature of *Unmada* is a deviation of *manas* (mind), *buddhi* (decision), *smriti* (memory), *sangya gyanam* (orientation and responsiveness), *bhakti* (desire), *sheela* (habit), *Cheshta* (activity), and *achaara* (conduct) as eight factors (either all of them or some of them).

Unmada are classified into five types. *Kaphaja Unmada* is one amongst them. It shares similarities with depression which are given in the table below:

Sr. No.	***Kaphaja unmada***	**Depression**
1.	*Sthaanamekadeshe*	Inactive/dull
2.	*Alpachankramana,* *Alpa cheshtha/ Cheshtha manda*	Psychomotor retardation
3.	*Alpa kathanam/ Manda vaak*	Talking less/Sad
4.	*Agnisaada/ Arochaka/ Alpa bhuk*	Loss of appetite, anorexia, anhedonia
5.	*Rahaskaamata, Vivikta priyata*	Social withdrawal
6.	*Bhibhatsatva/ Shaucha dvesha*	Ignoring personal hygiene, hating cleanliness
7.	*Sadana*	Fatigue, lassitude, lack of energy, body pain

8.	*Alpa mati*	Decreased cognitive functions/higher mental functions/difficulty in thinking, slowed thinking
9.	*Smriti vibhrama*	Poor memory

Table 1.4 Comparison of symptoms between Kaphaja unmada and depression (Gupta & Mamidi, 2015; Tubaki et al., 2021)

In the treatment of *kaphaja unmada*, *Shodhana* (purification by expelling the increased doshas) is done by using formulations such as *Kalyanaka ghrita, mahapaishachika ghrita, mahakalyanaka ghrita, siddharthaka ghrita* which has *Chandana, Vacha,Jatamansi* and *Karpura* as a content of the formulation.

4. Caenorhabditis elegans

Kingdom: Animalia
Phylum: Nematoda
Class: Chromadorea
Order: Rhabditida
Family: Rhabditidae
Genus: *Caenorhabditis*
Species: *C. elegans*

Caenorhabditis elegans is a small but well-differentiated free-living soil organism. It feeds on bacteria and is an important model system for biological research in many fields including genomics, cell biology, neuroscience and ageing (Leung et al., 2008). *C. elegans* is an ideal model for ageing and neurodegenerative diseases. (Peng et al., 2011)

Young larvae measure 0.25 mm long and adults 1 mm because of which they are viewed under a dissecting or compound microscope. The life cycle of *C. elegans* is about 2 to 3 weeks at 20°C, starting as eggs and going through four larval stages before

maturing. *C. elegans* has two sexes: male and hermaphrodite. Hermaphrodites can self-fertilize and produce hundreds of offspring. *C. elegans* consists of 12 chromosomes in hermaphrodites, five pairs of autosomes - two sex chromosomes, and 11 chromosomes in males, which have a single sex chromosome. They have 302 neurons that make up the *C. elegans* nervous system (Ann K. Corsi, 2015).

C. elegans are grown and maintained on agar plates seeded with bacteria *E. coli* OP50. The muscles of *C. elegans* are arranged in an unsegmented, tapering pattern and are connected to the hypodermis by a strong cuticle cover. For locomotion, it uses a series of opposing muscles. It crawls or swims through the lawn of bacteria with a characteristic sinusoidal pattern. Its trajectory is a random walk, and it goes towards food or potential mates (Donald L. Riddle, 1997)

Chemosensation

Caenorhabditis elegans has been used as a model organism to study chemotactic behavior in a variety of research contexts spanning diverse fields such as neurophysiology, neurobiology, and toxicology, and has played an important role in elucidating chemosensory mechanisms. *Caenorhabditis elegans* detects the presence of inorganic substances and solutes through a specialized set of chemosensory neurons, the amphid chemosensory phasmid, and inner labial neurons. Amphid neurons, in 11 pairs, are primarily responsible for detection. The detection of volatile and soluble substances requires different types of sensory neurons. *C. elegans* also perceives taste and smell through several families of chemoreceptors. Chemosensation has been extensively studied and the neural circuits and receptors involved have been well described by (Bargmann, 2006) In addition to odours and sweet substances, worms can detect a wide variety of substances, including the harmful gases oxygen and carbon dioxide, through a specialized set of sensory neurons that use guanylate cyclase as a molecular sensor (Bretscher et al., 2008)

The advantages of *C. elegans* as a model organism are short life cycle, compact genome, stereotypical development, ease of propagation, large number of progenies, transparent body and small size.

2. MATERIALS AND METHODS

2.1 Essential Oils-

Steam-distilled essential oils were ordered from Deve Herbes and Indus Valley. The details of which are as follows-

a. Pure camphor Essential oil (Deve Herbes, Batch ID- 0423)

b. Pure Calamus E O (Deve Herbes, Batch ID- 11/23)

c. Pure *Jatamansi* E O (Deve Herbes, Batch ID- 11/23)

d. Sandalwood E O (Indus Valley, Batch ID- 2625/8)

Absolute concentration was used for all the experiments.

Fig.2.1 Essential oils of *Jatamansi, Karpura, Vacha* and *Chandana*

2.2 *Caenorhabditis elegans* strains

The strains used in my study were:

1. *N2* Bristol WT strain

2. DA1814 *Ser-1* (ok345) strain

Mutant	Protein	Human Homologue	Role in Depression
DA1814	*Ser-1*	5-hydroxytryptamine receptor 2B (HTR2B)	agonism induces an SSRI-like response

Table 2.1 Information about knockout mutant used (Naß et al., 2021)

Both the strains were grown at 20˚ C for the experiments.

2.2.1 Culture and Media

All the buffers and media mentioned below were prepared according to Wormbook and WormAtlas (Theresa Stiernagle 1976, tit. Maintenance of *C. elegans*).

Preparing NGM plates-

Requirements- NaCl, Peptone, Agar, Distilled water, 1M MgSO4, 1M CaCl2, Cholesterol (5mg/ml of ethanol), potassium phosphate buffer (pH-6)

NGM media was prepared by maintaining sterile conditions. Plates were poured after they solidified, they were seeded with E. coli with appropriate volume. Plates were incubated for 16 hours at 37˚ C and then stored at 4˚C for 2-3 weeks.

E. coli (OP50) culture preparation

Requirements- Luria broth, distilled water

LB broth was prepared under sterile conditions. One colony of OP50 E. coli was inoculated to a test tube containing 2ml of LB broth, it was incubated for 6 hours at 37˚ C-120 rpm. Later, after 6 hours, 100µl of culture is transferred to the flask of LB and kept for incubation at 37˚C-120 rpm for 16 hours.

Bleaching

Requirements- Distilled water, 1M NaOH, NaClO (Bleach)

Day 1 adult worms Washed and collected from a petri plate to a falcon tube. After it settled the supernatant was discarded and 1ml of 20% alkaline hypochlorite solution was added. After the worms get dissolved add 4ml of autoclaved distilled water and centrifuge at 2000rpm for 2 minutes. Discarded the supernatant without disturbing the

pellet. Add distilled autoclave water to the pellet, mix well and depending on the number of eggs required aliquot it to the NGM plates.

2.3 Chemotaxis Assay in *C. elegans*

Chemotaxis was performed by (Margie et al., 2013). Briefly, Agar plates were prepared and divided into four equal quadrants, marked with ‘T’ for test and ‘C’ for control diagonally. Equidistant points, 2 cm apart, were marked on the plates. Two microliters of 100 mM sodium azide were applied to both ‘T’ and ‘C’. Worms were washed 4-5 times and collected, and approximately 150 worms were placed at the center. The drugs were applied to 'T' and 'C'. The plates were inverted and incubated at 20˚C for 1 hour. After 1 hour, the worms in each quadrant were counted, and the chemotaxis index was calculated using the formula: Chemotaxis Index (CI) = (Number of Worms in Both Test Quadrants – Number of Worms in Both Control Quadrants) / (Total Number of Scored Worms). An absolute concentration of four essential oils was used, and 1% Diacetyl (DA) dissolved in 90% ethanol was used as a positive control. Three independent trials were performed.

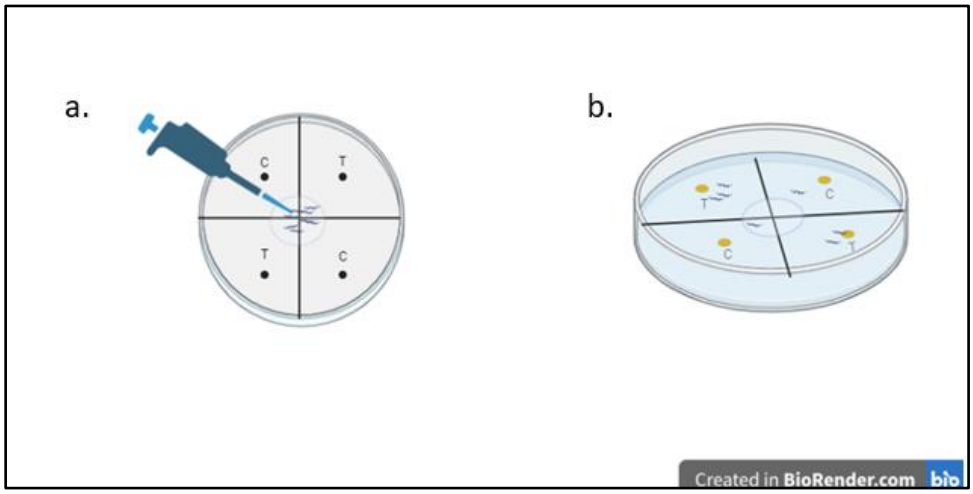

Fig. 2.2 Illustration of Chemotaxis assay. (a) Marked plate with T (test)- C (control) and worms at the centre. (b) Plate after an hour of incubation where the majority of worms attracted towards the test T.

2.4 Lifespan Assay in *C. elegans*

To determine the lifespan of the nematode's lifespan at 20˚C a modified protocol by (Solis & Petrascheck, 2011) was followed. Both N2 and Ser-1 strains were separately cultured on fresh NGM plates at least for 3 generations without starvation. 50, late L4 larvae were transferred to NGM plates containing OP50 E. coli. The time L4 larvae were transferred to NGM plates was defined as day 0. 1µl of the EO and control was applied freshly on each day to the lid of the plate. Live, dead and censored worms were counted on each day until all worms died. The worms were scored dead if they did not respond to external stimuli with a platinum-wire pick. The worms were transferred to fresh plates till they in their fertile plate and later were transferred on every alternate day. Everyday the worms were exposed to EO Three trials were done for each strain. Oasis software was used for analysing the data.

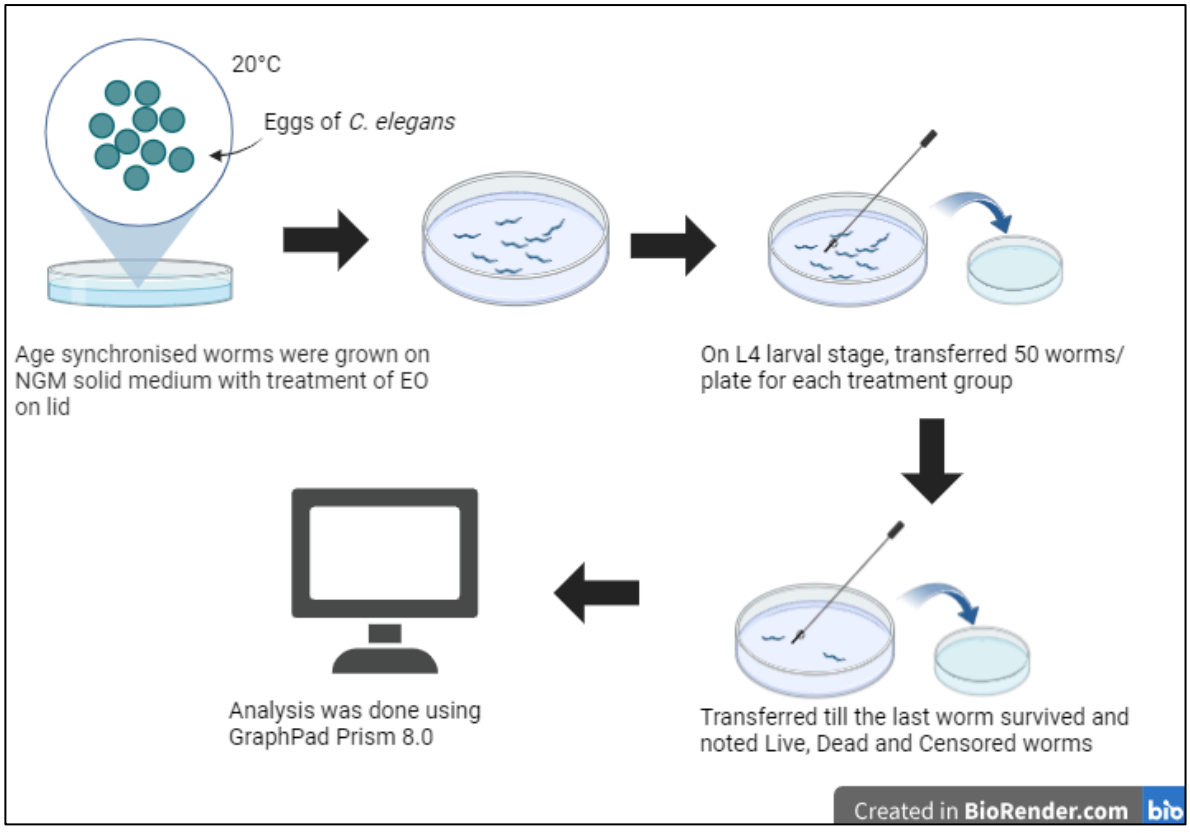
20°C
Eggs of C. elegans
Age synchronised worms were grown on NGM solid medium with treatment of EO on lid
On L4 larval stage, transferred 50 worms/ plate for each treatment group
Transferred till the last worm survived and noted Live, Dead and Censored worms
Analysis was done using GraphPad Prism 8.0
Created in BioRender.com

Fig.2.3 Illustration for Lifespan Assay

2.5 Oxidative Stress Assay in *C. elegans*

Oxidative stress is a type of stress caused due to free radicals in the cells of an organism. Reactive oxygen species which are produced from the redox cycling of paraquat are highly reactive compounds to degrade cellular macromolecules which induce lipid peroxidation and protein and nucleic acid degradation that leads to oxidative stress and finally cell death.

Here, acute oxidative stress was induced by the chemical Paraquat (100mM) and the effect of the drug was investigated using the protocol then the number of worms alive was scored to understand the effect of the drug on oxidative stress. It was assessed using protocol by (Possik & Pause, 2015). For this assay, N2 worms were grown on NGM plates with the treatment of EOs on the lid till the worms reached Day-1 adult. On the experiment day, five worms were transferred to each well in a 96-well plate for a particular group 6 replicas were done. The number of live worms in each well was determined by gently the worms with a pick to check for movement after an hour for 4-5 hours. Calculated the percentage of survival.

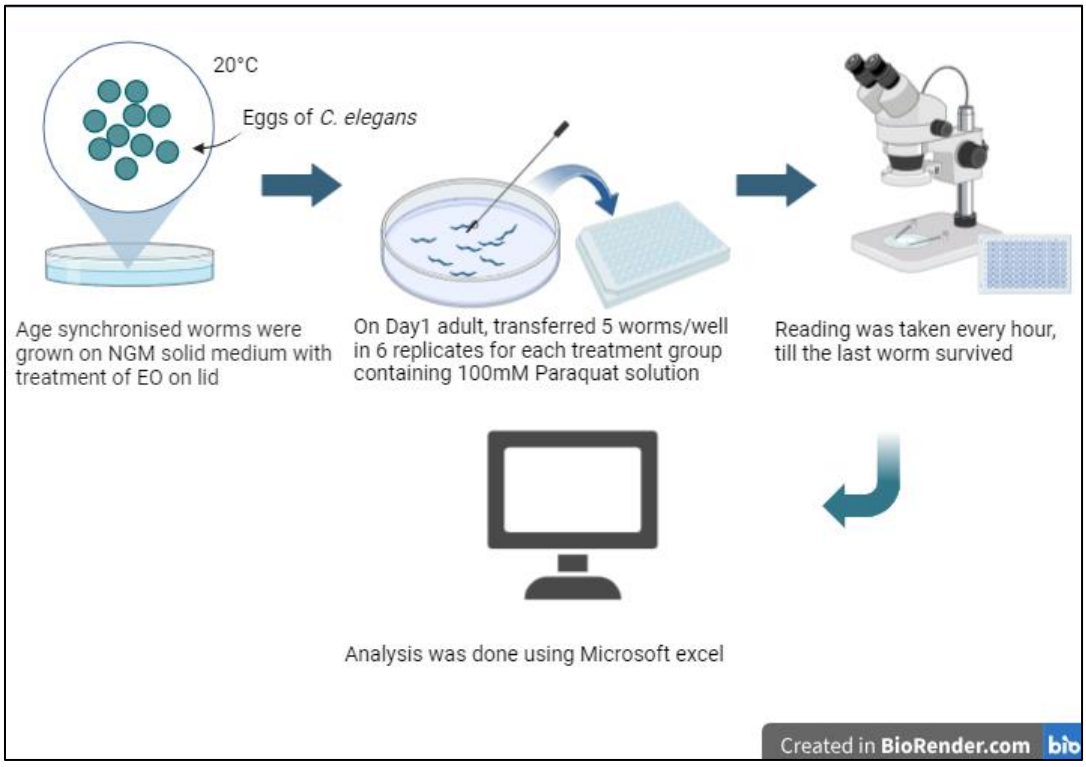
20°C
Eggs of C. elegans
Age synchronised worms were grown on NGM solid medium with treatment of EO on lid
On Day1 adult, transferred 5 worms/well in 6 replicates for each treatment group containing 100mM Paraquat solution
Reading was taken every hour, till the last worm survived
Analysis was done using Microsoft excel
Created in BioRender.com

Fig.2.4 Illustration for Oxidative stress using Paraquat

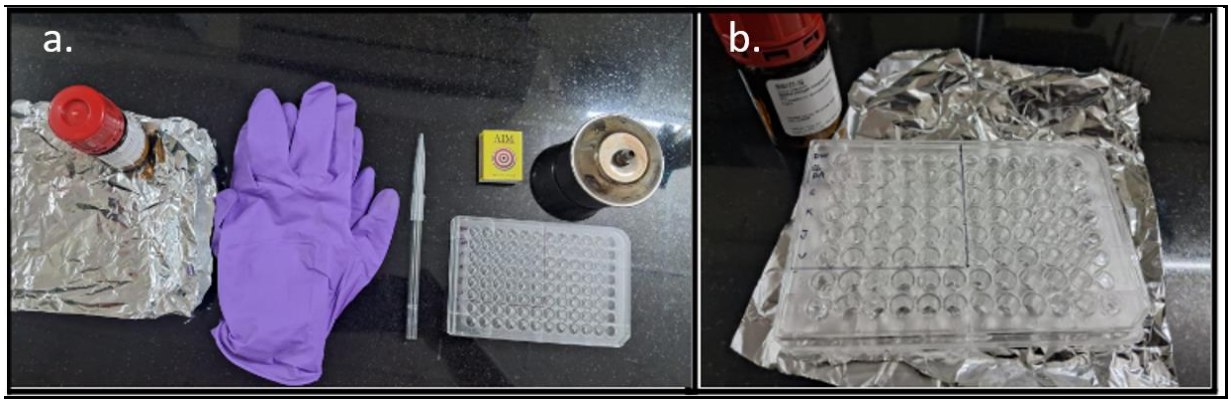

Fig. 2.5 Preparation for Oxidative stress assay

2.6 Fecundity

To observe egg laying in *C. elegans* the protocol by (Lee & Kang, 2017) was slightly modified and used. The EO was applied to the lid and the worms were exposed to it since the egg stage. Late L4 were picked and transferred to a fresh NGM plate, this was repeated for 7 days and the number of eggs laid were counted. Three independent trials were performed.

2.7 RNA isolation

The worms were washed and collected in labelled 1.5 ml Eppendorf tubes. TRIzol reagent was added according to the pellet amount (100-200). The centrifuge was set for fast cooling at 4°C. The worms were homogenized thoroughly. Chloroform was added in a 1:1 ratio with TRIzol, and the mixture was vortexed for 30 seconds. The tubes were allowed to rest at room temperature for 3 minutes, then centrifuged at 14,000 rpm for 15 minutes. The upper transparent phase was carefully transferred to newly labelled DEPC-treated Eppendorf tubes. Double the volume of isopropanol was added to the transferred phase and mixed well. The tubes were centrifuged at 14,000 rpm for 15 minutes. The supernatant was discarded, and the pellet was retained. 400 µl of 70% DEPC ethanol (prepared with DEPC-treated water) was added to the pellet. The pellet was freed from the tube by gentle agitation. The tubes were centrifuged at 14,000 rpm for 10 minutes. The supernatant was carefully removed without disturbing the pellet. This step was repeated once more. The tubes were allowed to air dry to remove

ethanol. Finally, 10-15 µl of DEPC-treated water was added to dissolve the RNA pellet. The RNA was quantified using a Nanodrop spectrophotometer.

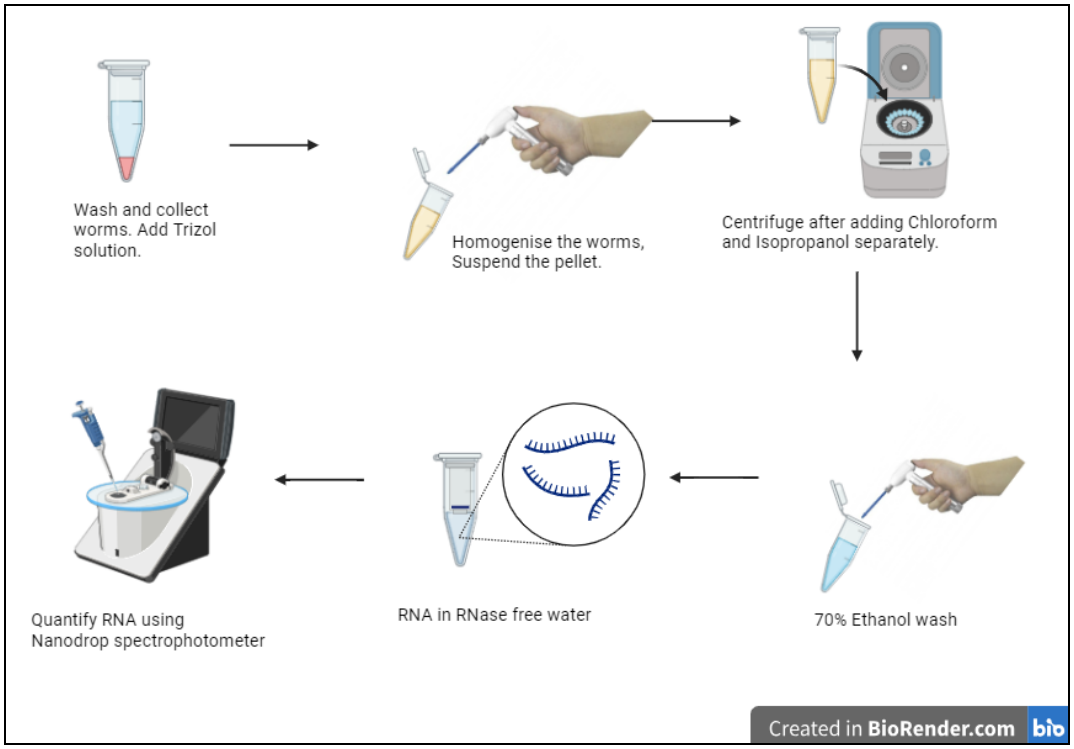
Wash and collect worms. Add Trizol solution.
Homogenise the worms, Suspend the pellet.
Centrifuge after adding Chloroform and Isopropanol separately.
70% Ethanol wash
RNA in RNase free water
Quantify RNA using Nanodrop spectrophotometer
Created in BioRender.com
bio

Fig.2.6 RNA isolation procedure

2.8 cDNA preparation

The RNA was quantified to assess purity. The volume required for 500 ng of cDNA and the volume of autoclaved distilled water was calculated. A Master Mix was prepared according to the protocol of TaKaRa in a 1.5 ml tube. 3.5 µl of the Master Mix was added to each sample. Water, RNA, and 3.5 µl of Master Mix were added sequentially to PCR tubes. The tubes were vortexed, and the samples were placed in labelled PCR tubes for synthesis and then stored at -20°C.

2.9 Sensory (Odour) Analysis of Selected EO on Human Volunteers

The sensory analysis only for the odour of EOs was performed using hedonic scaling which refers to a 9-point scale, with values from extremely unpleasant- neutral- to extremely pleasant in human volunteers. The study was blinded Ten healthy volunteers

participated and they were allowed to smell one EO per day so that there was a washout period of a particular EO. On the tailor-made questionnaire which had a Hedonic Scale, Intensity and Attributes were given to volunteers. The workflow of this is shown by Fig. 2.7.

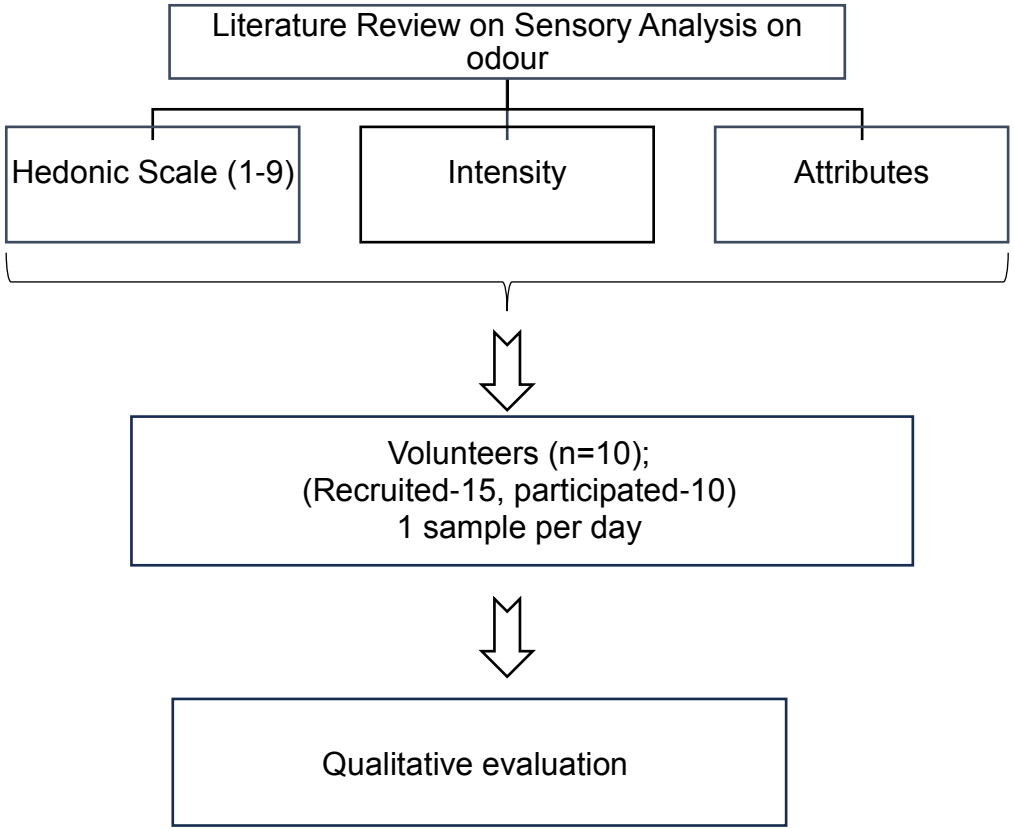

Fig. 2.7 Workflow for Sensory analysis of odour for human volunteers

2.10 Data analysis

Data analysis was done by using T-test for Chemotaxis assay, Oxidative stress assay and Sensory analysis. For lifespan experiments log-rank (Mantel-Cox) method is used to test the null hypothesis and calculate P-values. Software such as OASIS 2, Prism GraphPad 8.0.1 and Microsoft Excel was used to analyse data.

3. RESULTS AND DISCUSSION

3.1 Selected aromatic EO except *Karpura* are attractants for wild-type and Serotonin mutants of *C. elegans*

Since selected formulations are odours used in Ayurvedic treatments or therapies, we evaluated their effect using a well-standardized chemotaxis assay to study the effect of volatiles on *C. elegans* behaviour and other physiological parameters.

It was observed that *Chandana, Jatamansi* and *Vacha* EOs act as attractants and worms get repelled from *Karpura*. The extent of attraction or repulsion is indicated by the chemotaxis index (CI). In wild-type (N2) strain worms, *Chandana* acts as a strong attractant (CI 0.81) followed by *Jatamansi* (CI 0.44) and *Vacha* (CI 0.36) and *Karpura* came up as a repellant with (CI -0.38) (Fig. 3.1)

We also assessed chemotaxis in Ser-1 serotonin mutant, which is a model to study depression. A Similar trend was observed with CI of 0.56, 0.44, and 0.40 for *Chandana, Vacha* and *Jatamansi* respectively. *Karpura* had a CI of -0.20, but the amplitude of the CI was significantly reduced (Fig. 3.2). 1% Diacetyl is a known attractant which was used as a positive control.

The trend for both the graphs of N2 and Ser-1 were the same, but there was a change in amplitude in bars of *Chandana* and *Karpura*-treated worms in Ser-1. It might be because these EOs might be working through the Serotonin pathway which is involved in mental health disorders like Depression.

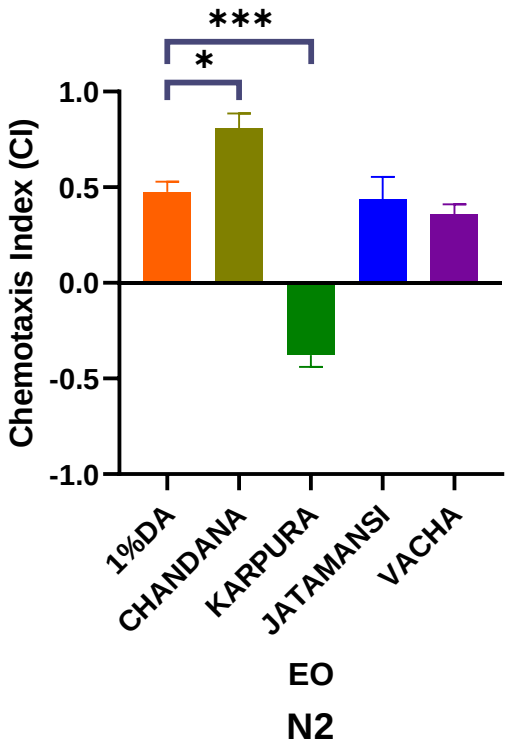

Fig. 3.1 Chemotaxis Index for N2

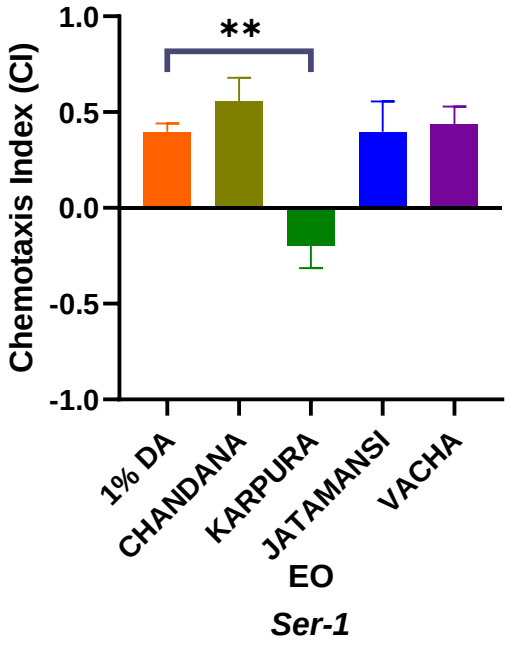

Fig. 3.2 Chemotaxis Index for Ser-1

	N2	Ser-1
DIACETYL 1%	0.476667	0.396667
CHANDANA	0.806667	0.556667
KARPURA	-0.37667	-0.19667
JATAMANSI	0.436667	0.396667
VACHA	0.356667	0.436667

Table 3.1 Comparison of CI between N2 and Ser-1

3.2 *Chandana* and *Vacha* EO increase mean lifespan in Ser-1 mutants and no changes in lifespan are seen in N2 worms.

The findings indicated no increase or decrease in the lifespan of wild-type worms and showed a significant increase in lifespan with Ser-1 mutants on exposure to *Chandana* ($p=0.0015$ **) and *Vacha* ($p=0.0432$ *). As observed in Ser-1 mutants treated with *Chandana* and *Vacha*, increased lifespan. (Fig. 3.4 (b) and (e)). This might be suggestive of, *Vacha* and *Chandana* contain bioactive compounds that have beneficial effects on the longevity of *C. elegans*, particularly in the absence of the Ser-1 receptor. The positive impact on lifespan could be due to the presence of antioxidant or anti-inflammatory bioactive in both the EOs that enhance stress resistance and promote cellular health. The absence of the Ser-1 receptor might alter the worm's metabolic and stress response pathways, making them more responsive to these beneficial bioactives.

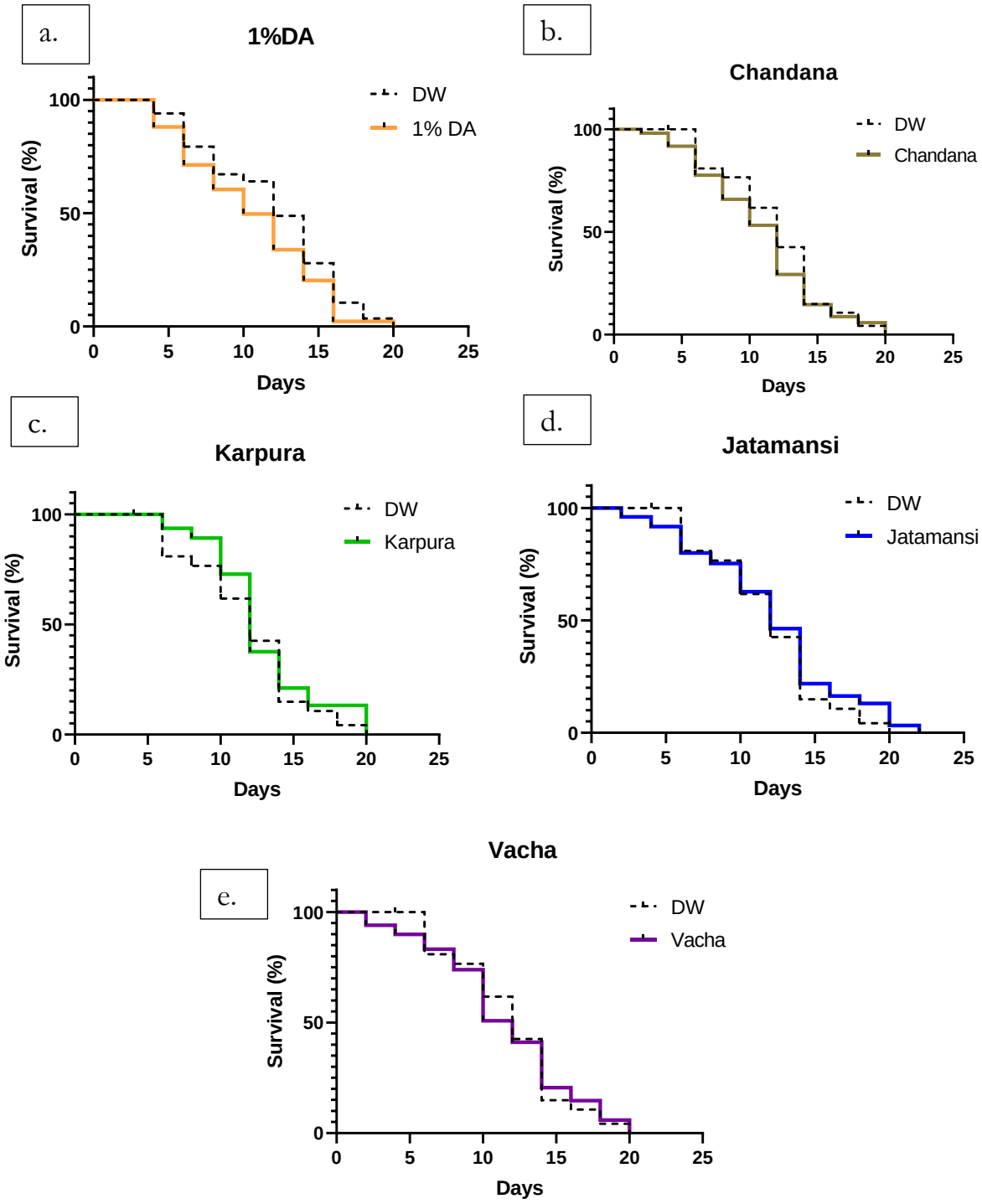

Fig.3.3 Lifespan of N2 treated with samples

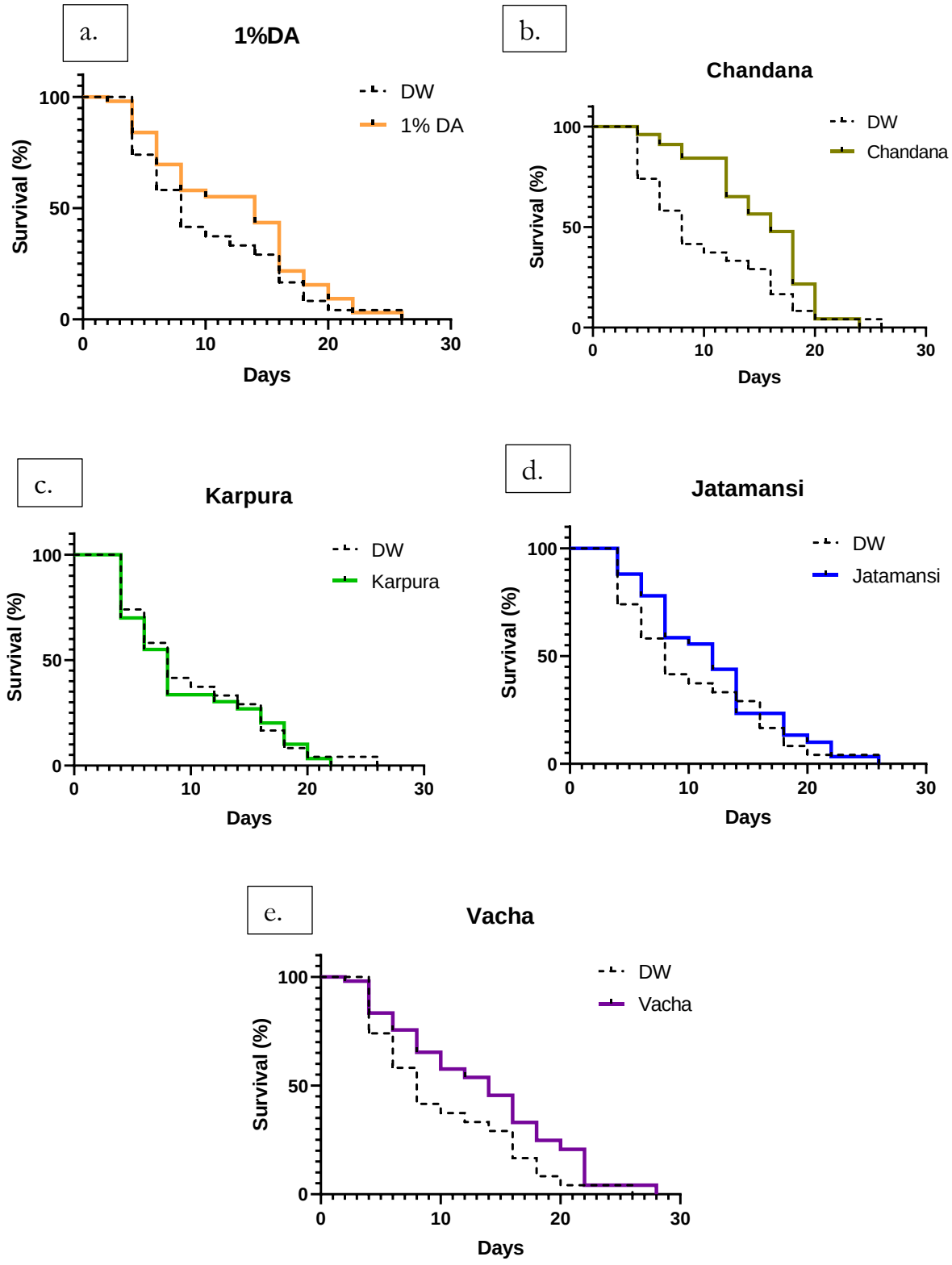

Fig.3.4 Lifespan of Ser-1 treated with samples

3.3 Worms treated with *Chandana* can tolerate higher oxidative stress

Stress resilience is a good indicator of wellness and health span. According to Ayurveda, the effect of *Sugandhi Dravya* is to give pleasure and happiness, which can contribute to the wellness of an individual.

On exposure to Paraquat after giving treatment for 72 hours it was observed that, to control all the EOs were extending the tolerance to Oxidative stress. At 1 hour, the worms treated with EOs showed not much difference from the control.

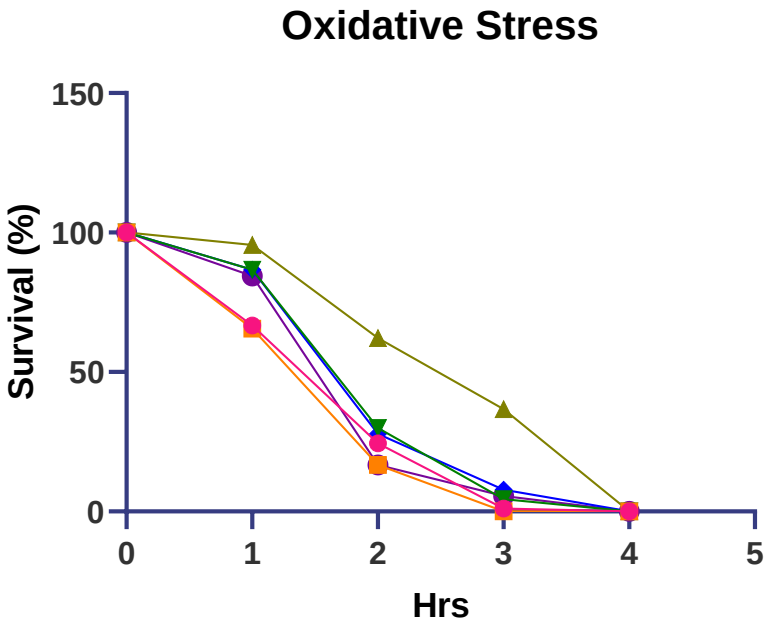

Fig.3.5 Oxidative stress with all the samples

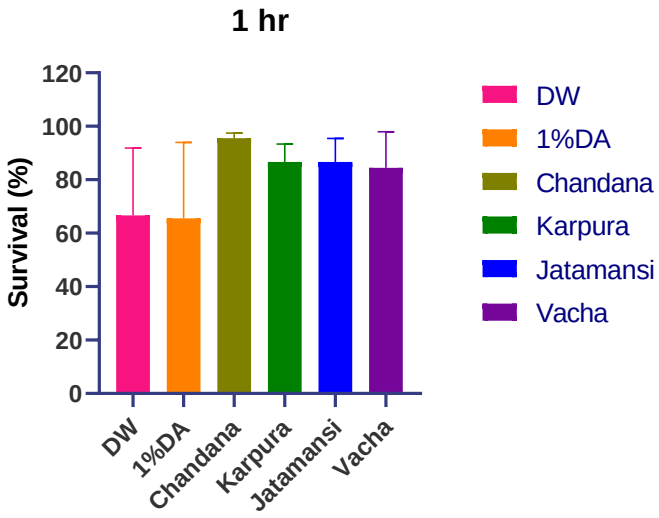

Fig.3.6 Oxidative stress after at 1 hour for all the sample

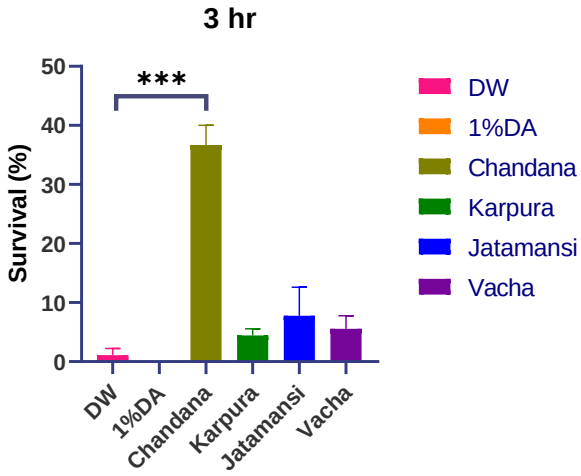

Fig.3.7 Oxidative stress after 3 hours for all the sample

It was observed at 3 hours that, the worms in all four EOs lived longer than the control worms($p=0.006$). This might be because the worms have evolved to protect themselves from oxidative damage by complex mechanisms that detoxify ROS.

3.4 Egg laying increases in N2 worms on exposure to *Vacha*

Reproductive capacity is an indicator of health. Further, in C elegans fecundity, especially egg-laying has a connection with serotonin. Thus, we checked the effect of exposure to *Sugandhi dravya* on fecundity.

It was observed that upon treatment with *Vacha*, the fecundity of N2 worms, as represented by (Fig. 3.8) showed an increase in the total number of eggs laid in 7 days in comparison with the control, 1% DA and *Chandana* treated groups.

These early results were surprising. We need to investigate more into the trends showing an increase in fecundity by *Vacha* EO exposure.

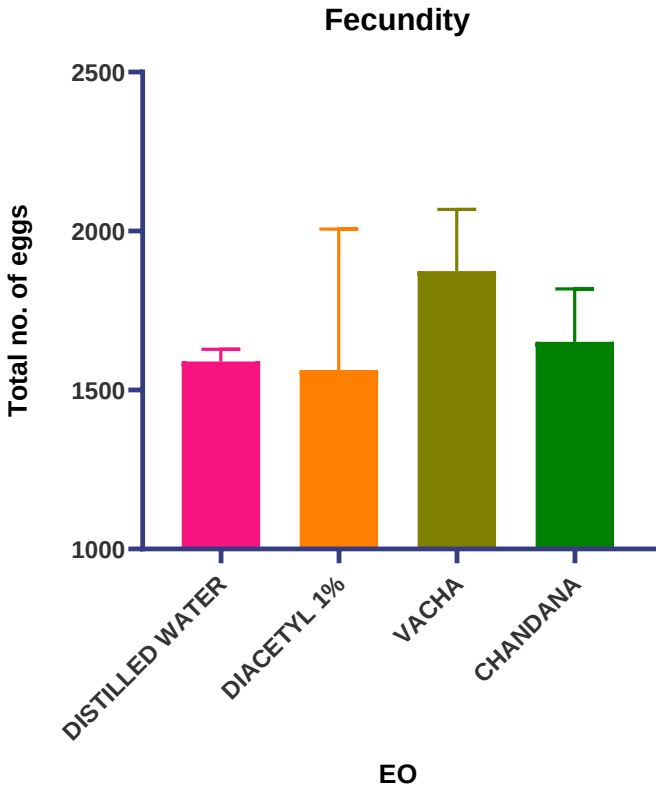

Fig.3.8 Total number of eggs laid by 12 N2 worms in 7 days

3.5 RNA Isolation

RNA isolation was done of wildtype worms we observed good quantity and purity of the sample as shown in Table 3.1

Samples	RNA (ng/µL)	A260/A280	A260/A230
DW1	1893.3	2.25	1.93
DW2	1054.3	2.21	2.13
Chandana 1	758	2.25	1.94
Chandana 2	663	2.26	2.2

Table 3.2 Quantity and purity of samples

3.6 Sensory Analysis in Human Volunteers

The volunteers liked all EOs; *Chandana* being favoured the most

Based on whether or not the smell perceived by the participants was liked or not the evaluation was done using a 9-point hedonic scale. It was observed that participants liked *Chandana* the most (8.2) followed by *Karpura* (6.9), *Vacha* (6.5), *Jatamansi* (5.8), 1% DA (5.2) and Distilled water (5.2) (Fig 3.9). It was observed that in two parameters-sense of good feeling and headache, the odour of all EOs gave a sense of good feeling to the participants. Two participants experienced slight headache just for 15-20 minutes (Fig 3.11). It was observed that most of the volunteers had a sense of good feeling after sniffing the odour of essential oils, *Chandana* was favoured the most for this feeling (Fig. 3.10). As the smell of these drugs are acceptable and as they have shown a positive effect in the extension of lifespan, positive response to oxidative stress and increase in fecundity it can be studied further on higher model organisms.

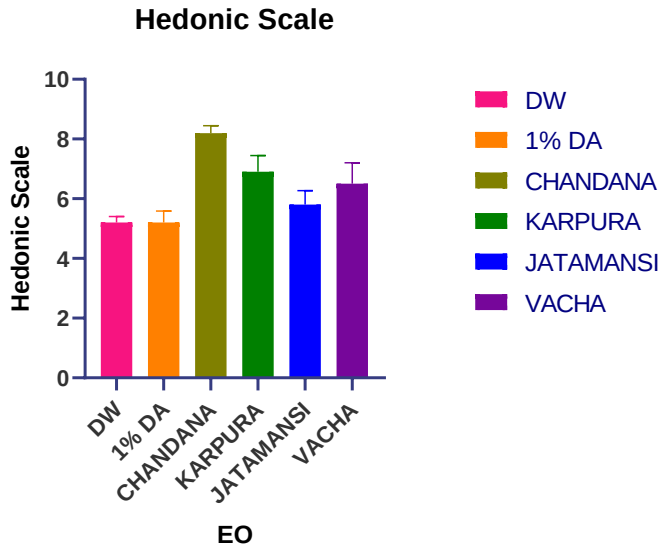

Fig.3.9 Evaluation of EOs using hedonic scale for human participants

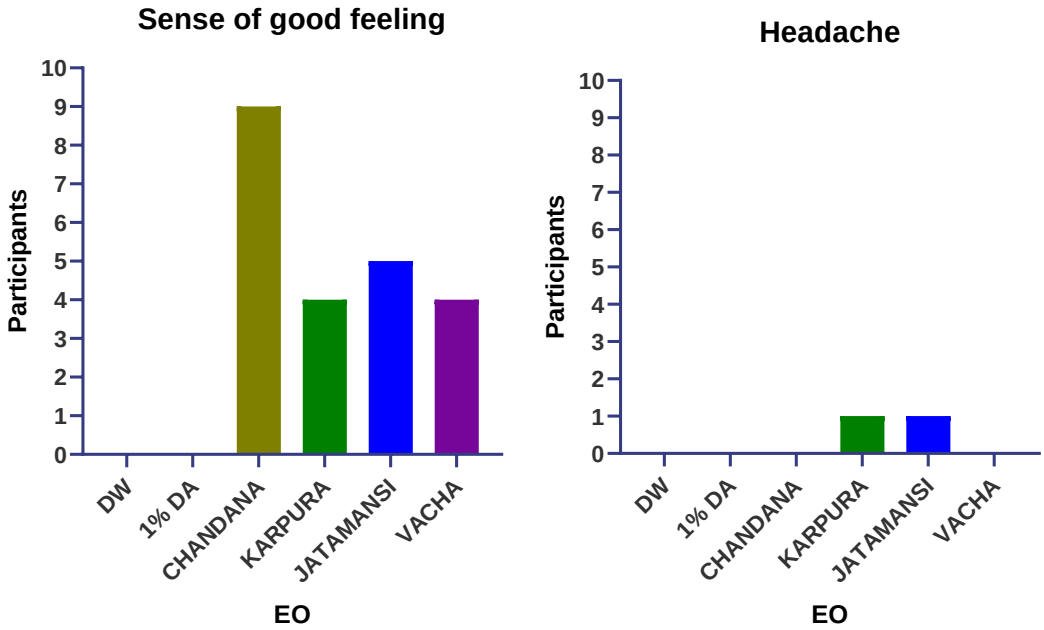

Fig 3.10 Frequency graph for two of the attributes from the questionnaire. Sense of good feeling and Headache

4. **CONCLUSION**

- The four aromatic drugs were selected considering their traditional use in treating *Kaphaja unmada*, which shares symptoms with depression disorders. Evaluation of the effect of these odorants in the *C. elegans* model is that these drugs demonstrated an ability to reduce stress and extend health span and lifespan, particularly under serotonin-deprived conditions that mimic depression. The Essential oil of *Chandana* was found to be most effective. Additionally, *Vacha* extension in lifespan in *Ser-1* mutants also showed a notable effect in enhancing fecundity.

- In the part of the study involving human volunteers, sensory analysis using a hedonic scale in healthy adults indicated that *Chandana* is the most preferred and safest odorant among the tested oils, as it was the 'most liked' odorant with a hedonic score of 8.2. It did not cause any side effects such as nausea or headache.

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APPENDIX

1. **Progress Report 1**

Title-

Exploring the antiaging and nootropic potential of aromatic essential oils using *Caenorhabditis elegans* models.

Objectives-

1. Selection of the essential oils (EO) based on Ayurvedic and modern literature as well as practice.
2. Evaluation of differential effect of aromatic essential oils on longevity of *C. elegans.*
3. To study the effect of essential oils on chemosensory behavior in disease and wild type C elegans models.
4. To investigate the effect of aromatic essential oils on the stress model of *C. elegans.*
5. Sensory analysis to understand aroma preference of the selected essential oils in the lay human population.

Progress made in objectives-

- Objective 1
    - Selection of oils.
    - Quality of oil procured (steam distilled and extracted from pure plant material)
    - In Dravyaguna sangraha according to the definition of 'sugandh' (aromatic), one that gives a sense of pleasure and satisfaction; these improve appetite. The therapeutic effects of these drugs vary.
    - 4 aromatic plants for the project are-
        - *Vacha* (*Acorus calamus* Linn)
        - *Karpura* (*Cinnamomum camphora (L.) Nees & Eberm*
        - *Jatamansi* (*Nardostachys Jatamansi (D.Don) DC)*
        - *Chandana* (*Santalum album* L.)

- The use of Aromatic drugs in Ayurvedic literature at various places in Samhita.
- Literature review on effects of aromatic drugs on *C. elegans* in different research papers, listing some:

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC8036369/ ,
https://www.sciencedirect.com/science/article/abs/pii/S0944711321001409,
https://www.sciencedirect.com/science/article/abs/pii/S0278691521007961

- Objective 3
  - Standardization of treatment protocol: Growing *C. elegans* in the presence of essential oil of 100% concentration.
  - Sensory analysis Using Chemotaxis assay

Skills learned-

- Maintaining plate of *C. elegans.*
- Learning to pick worms.
- Media preparation
- Pouring of plates
- Preparation of Luria Broth
- Preparing E. coli culture
- Bleaching worms

Challenges faced-

- Deciding the *C. elegans* strains to use
- Finding authentic vendors for steam distilled-procured from plant material essential oil.
- Writing protocol (IEC etc is difficult so dropped the clinical study, but will do the sensorial analysis with a single time point).

2. **Progress Report 2**

Title-

Exploring the antiaging and nootropic potential of aromatic essential oils using *Caenorhabditis elegans* models.

Objectives -

1.Selection of the essential oils (EO) based on Ayurvedic and modern literature as well as practice.

2.Evaluation of differential effect of aromatic essential oils on longevity of *C. elegans.*

3. To study the effect of essential oils on chemosensory behavior in disease and wild type C elegans models.

4. To investigate the effect of aromatic essential oils on the stress model of *C. elegans.*

5. Sensory analysis to understand the aroma preference of the selected essential oils in the lay human population.

Progress made-

- Effect of selected aromatic essential oils on chemosensory behavior was evaluated through chemotaxis assay in wild type young adult worms.
- Effect of selected aromatic essential oils on egg laying was evaluated through fecundity in wild type young adult worms. One trial was performed and other trials are ongoing.
- Literature review on Lifespan assay done, planned lifespan assay from February.
- Literature review on phenotypic assays- ABC type movement, fecundity done.

Skills learned-

- Picking worms
- Preparing the reagents
- Counting of eggs.
- Setting experiment in duplicates for chemotaxis.

- Setting Fecundity experiment.

Challenges faced-

- While picking worms they either died or got injured.
- Literature on volatile compounds in essential oils.
- Literature survey for primers to be used for q-PCR.
- Obtaining strains.

3. Progress Report 3

Title-

Exploring the antiaging and nootropic potential of aromatic essential oils using *Caenorhabditis elegans* models.

Objectives-

1. Selection of the essential oils (EO) based on Ayurvedic and modern literature as well as practice.
2. Evaluation of differential effect of aromatic essential oils on longevity of *C. elegans*.
3. To study the effect of essential oils on chemosensory behavior in disease and wild type *C. elegans* models.
4. To investigate the effect of aromatic essential oils on the stress model of *C. elegans.*
5. Sensory analysis to understand aroma preference of the selected essential oils in the lay human population.

Progress made-

- The effect of selected aromatic essential oils on chemosensory behavior was evaluated through chemotaxis assay in wild-type and Serotonin mutant (Ser-1 and Ser-4) young adult worms. Three trials were performed.
- Egg laying of wild-type, Serotonin mutants ie. Ser-1 and Ser-4 are evaluated through fecundity to select which mutant to be used. Two trials were done.

- Planning to experiment with the essential oils with Ser-1 mutants.
- The effect of selected aromatic essential oils on longevity was evaluated through Lifespan assay. Second trial ongoing with wildtype of worms.
  Planned to set up Ser-1 lifespan in April.
- Literature review on Stress assays.
- Literature review on sensory analysis.

Skills learned-

- Picking worms
- Preparing the reagents.
- Counting of eggs.
- Conducted experiments in duplicates for chemotaxis.
- Conducted Fecundity experiment.
- Conducted lifespan assay.
- Data analysis in Excel.
- Learned to operate Oasis software for analysis of lifespan.

Challenges faced-

- Selection of which strain to be used.
- Literature survey for primers to be used for q-PCR.
- Analysis of the data.